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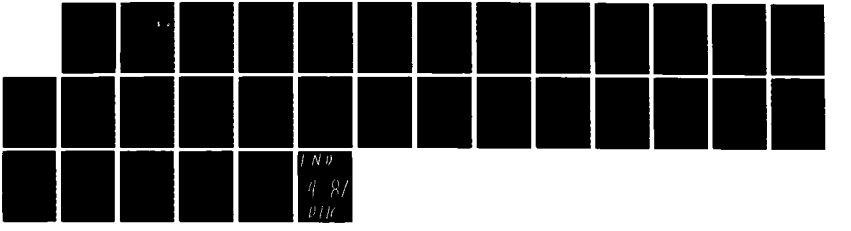
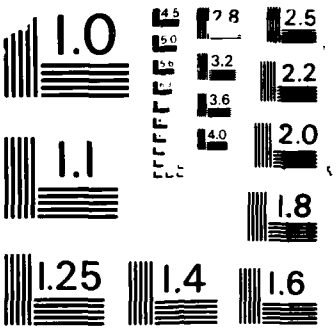
UNCLASSIFIED DAMD17-82-C-2021 F/G 6/13 NL





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STUDIES ON THE IMMUNOCHEMICAL TECHNIQUES FOR DETECTION OF SELECTED FUNGAL AND DINOFLAGELLATE TOXINS

Annual/Final Report

August 15, 1986

F. S. Chu, Ph.D.

Submitted by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2021

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Wisconsin; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Food Research Institute and Department of Food Microbiology and Toxicology, Madison, Wisconsin 53706	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-82-C-2021
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63763A; PROJECT NO. 3M263, 763D807; TASK NO. AG; WORK UNIT ACCESSION NO 032

11. TITLE (Include Security Classification)
(U) Studies on the Immunochemical Techniques for Detection of Selected Fungal and Dinoflagellate Toxins

12. PERSONAL AUTHOR(S)
F. S. Chu, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final*	FROM 11/1/81 TO 7/15/86	1986 August 15	26

16. SUPPLEMENTARY NOTATION
*Annual for the period Sep 85-Apr 86
Final for the period Nov 81-Jul 86

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
antibody, immunoassay, saxitoxin, T-2 toxin, trichothecenes

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The progress during the entire contract (Nov. 1, 1981 to July 15, 1986) is summarized as follows: (i) conditions for improving antibody production against T-2 toxin were studied. T-2-HS-BSA which had 10-15 mole of T-2 per mole of BSA was found to be a better immunogen than T-2-HG-BSA; (ii) methods for the production of antibodies in rabbits against diacetoxyscirpenol (DAS), deoxyverrucarol (DOVE), acetyl-deoxynivalenol (Ac-DON) and T-2 toxin metabolites including 3'-OH-T-2, HT-2, and T-2 tetraol tetra-acetate were developed. These antibodies were highly specific to their respective trichothecenes or metabolites that were used in the conjugation; (iii) a new improved method for the production of antibodies against T-2 toxin and DAS in rabbits was developed. More stable protein conjugates of carboxymethyl derivatives (CMO) of these toxins were used in the immunization;

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Mrs. Judy Pawlus	301-663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

(iv) a generic-type antibody which cross-reacted with most type A trichothecene mycotoxins was obtained after immunization of rabbits with 3-Ac-NEOS-HS-BSA; (v) saxitoxin antibody was raised after immunizing rabbits with STX-HCHO-BSA. The antibody showed high specificity to STX with some cross-reaction with decarbamoyl-STX (56%) and neo-STX (16%); (vi) a number of tritiated trichothecene mycotoxins of high specific radioactivity were prepared in our laboratory. These labelled toxins have been used as marker ligands in RIA of these mycotoxins as well as for metabolic studies. With the combination of the high specific radioactive T-2 toxin and high-titer antibody, as little as 25 pg of T-2 toxin can be detected by the RIA in each assay; (vii) With the availibility of different antibodies, sensitive ~~(0.1-0.5 ng/assay)~~ and specific RIAs for these mycotoxins (DAS, DOVE, and DON) were developed; (viii) a direct competitive ELISA for DAS with a detection limit of 25 pg per assay was developed. However, because of interferring substances in the sample matrix, the minimal detection levels for DAS in wheat and corn by RIA and ELISA are found to be 25 and 50 ppb, respectively; (xi) an indirect ELISA which permits to detect ~~0.2 to 1 ppb of~~ T-2 toxin in urine, serum and milk was also established; ~~Using both anti-T-2 and anti-HT-2 antibodies,~~ an indirect ELISA for simultaneous analysis of HT-2 and T-2 toxin in urine was also established; (x) an indirect ELISA with detection limits of 25 pg/assay for analysis of saxitoxin (STX) was developed. The detection limit for analysis of STX in mussels and clams by this method was found to be around 50-100 ppb without sample treatment; (xi) antibodies against T-2 toxin and DAS have been used to localize T-2 toxin in different tissues and organs of mice by a immunohistochemical technique developed in our laboratory; (xii) the ability of producing T-2 toxin and DAS by 18 Fusaria was studied by RIA. Among 13 F. sporotrichioides tested, all but one were found to be T-2 toxin or/and DAS producers. The amount of toxin(s) produced by each species varied considerably with species and incubation temperatures. Three F. graminearum and 2 F. chlamydosporum cultures did not produce any T-2 toxin and DAS; (xiii) limited efforts for production of monoclonal antibodies for STX and T-2 toxin were made. A stable clone for T-2 toxin was obtained by Dr. Hunter with our supply of appropriate reagents; (xiv) the metabolism of T-2 toxin in vitro was found to be affected by esterase inhibitors greatly. Thus, an efficient method for the preparation of large quantities of 3'-OH-T2 toxin, 3'-OH-HT-2, 3'-OH-acetyl-T-2 and 3'-OH-T-2 triol was developed; (xv) routine production of antibodies against T-2 toxin and DAS continued throughout the contract with the new T-2 toxin and DAS derivatives; (xvi) the immunochemical reagents prepared for and delivered to the USAMRIID are summarized.

FOREWARD

The following is a final report of the work performed under contract No. DAMD17-82-C-2021, during the period of Nov. 1, 1981 to July 15, 1986. The work was carried out at the Food Research Institute of the University of Wisconsin-Madison, under the direction of the principal investigator, Dr. F. S. Chu and co-principal investigator, Dr. E. J. Schantz. The contract officer is Dr. Robert W. Wannemacher, Jr.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

ABSTRACT

The progress during the entire contract (Nov. 1, 1981 to July 15, 1986) is summarized as follows: (i) conditions for improving antibody production against T-2 toxin were studied. T-2-HS-BSA which had 10-15 mole of T-2 per mole of BSA was found to be a better immunogen than T-2-HG-BSA; (ii) methods for the production of antibodies in rabbits against diacetoxyscirpenol (DAS), deoxyverrucarol (DOVE), acetyl-deoxynivalenol (Ac-DON) and T-2 toxin metabolites including 3'-OH-T-2, HT-2, and T-2 tetraol tetra-acetate were developed. These antibodies were highly specific to their respective trichothecenes or metabolites that were used in the conjugation; (iii) a new improved method for the production of antibodies against T-2 toxin and DAS in rabbits was developed. More stable protein conjugates of carboxymethyl derivatives (CMO) of these toxins were used in the immunization; (iv) a generic-type antibody which cross-reacted with most type A trichothecene mycotoxins was obtained after immunization of rabbits with 3-Ac-NEOS-HS-BSA; (v) saxitoxin antibody was raised after immunizing rabbits with STX-HCHO-BSA. The antibody showed high specificity to STX with some cross-reaction with decarbamoyl-STX (56%) and neo-STX (16%); (vi) a number of tritiated trichothecene mycotoxins of high specific radioactivity were prepared in our laboratory. These labelled toxins have been used as marker ligands in RIA of these mycotoxins as well as for metabolic studies. With the combination of the high specific radioactive T-2 toxin and high-titer antibody, as little as 25 pg of T-2 toxin can be detected by the RIA in each assay; (vii) With the availibility of different antibodies, sensitive (0.1-0.5 ng/assay) and specific RIAs for these mycotoxins (DAS, DOVE, and DON) were developed; (viii) a direct competitive ELISA for DAS with a detection limit of 25 pg per assay was developed. However, because of interferring substances in the sample matrix, the minimal detection levels for DAS in wheat and corn by RIA and ELISA are found to be 25 and 50 ppb, respectively; (xi) an indirect ELISA which permits to detect 0.2 to 1 ppb of T-2 toxin in urine, serum and milk was also established. Using both anti-T-2 and anti-HT-2 antibodies, an indirect ELISA for simultaneous analysis of HT-2 and T-2 toxin in urine was also established; (x) an indirect ELISA with detection limits of 25 pg/assay for analysis of saxitoxin (STX) was developed. The detection limit for analysis of STX in mussels and clams by this method was found to be around 50-100 ppb without sample treatment; (xi) antibodies against T-2 toxin and DAS have been used to localize T-2 toxin in different tissues and organs of mice by a immunohistochemical technique developed in our laboratory; (xii) the ability of producing T-2 toxin and DAS by 18 Fusaria was studied by RIA. Among 13 F. sporotrichioides tested, all but one were found to be T-2 toxin or/and DAS producers. The amount of toxin(s) produced by each species varied considerably with species and incubation temperatures. Three F. graminearum and 2 F. chlamydosporum cultures did not produce any T-2 toxin and DAS; (xiii) limited efforts for production of monoclonal antibodies for STX and T-2 toxin were made. A stable clone for T-2 toxin was obtained by Dr. Hunter with our supply of appropriate reagents; (xiv) the metabolism of T-2 toxin in vitro was found to be affected by esterase inhibitors greatly. Thus, an efficient method for the preparation of large quantities of 3'-OH-T2 toxin, 3'-OH-HT-2,

3'-OH-acetyl-T-2 and 3'-OH-T-2 triol was developed; (xv) routine production of antibodies against T-2 toxin and DAS continued throughout the contract with the new T-2 toxin and DAS derivatives; (xvi) the immunochemical reagents prepared for and delivered to the USAMRIID are summarized.

I. INTRODUCTION

The trichothecene mycotoxins are a group of toxic secondary fungal metabolites, produced by a number of fungi in the genera Fusarium, Trichoderma, Myrothecium and Stachybotrys which are frequently isolated from foods and feeds. Among this group of mycotoxins, T-2 toxin is the most toxic and has been found to be one of the most potent protein inhibitors. It has been found to be associated with several natural outbreaks of mycotoxicoses in both animals and humans, including implication as the cause of alimentary toxic aleukia (ATA), a disease which caused many fatalities among people who consumed bread made from over-wintered moldy wheat during World War II in the Orenberg district in the USSR. Diacetoxyscirpenol (DAS), a trichothecene discovered in the early sixties, which has been shown to possess antibiotic and antitumor effects, is also highly toxic. Recent studies indicated that T-2 toxin, DAS and related trihcothecenes were present in the "yellow rain" samples obtained from Southeastern Asia. Saxitoxin (STX) is one of the major toxin and most potent in a group of toxins involved in paralytic shellfish poisoning. The toxin is predominately produced by the dinoflagellate Gonyaulax catenalla and is primarily isolated from toxic mussels, clams and other marine animals in waters inhibited by Gonyaulax. Saxitoxin is extremely toxic and the amount that causes death in humans is estimated to be about 0.5 to 4.0 mg. Therefore, the presence of trichothecene mycotoxins and STX in foods, feeds and other environments is considered to be potentially hazardous to humans and animals.

Mycotoxins in the trichothecene family and STX, however, do not have a chromophore group which may provide a simple and sensitive method for quantitation of the toxins in foods, feeds, and also biological fluids. There is a need to develop a rapid method for monitoring these toxins in the environment and in body fluids. In view of the specificity of antigen-antibody interaction, studies in our laboratory (publication No. 3, 14, 15) and others have led to the production of specific antibodies against a number of mycotoxins including T-2 toxin. The goal of this contract was to develop a method for the production of antibodies against several selected trichothecene mycotoxins and dinoflagellate phytotoxins and subsequently to develop a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) for toxin determination as well as to use these antibodies as prophylactic agents. To achieve the main objective, the following specific tasks were planned:

(a) development of methods for conjugation of saxitoxin (STX) and its related dinoflagellate toxins to protein carriers;
(b) development of methods for conjugation of trichothecene mycotoxins to protein carriers;
(c) elicit antibodies against the toxin-protein conjugates;
(d) development and refinement of RIA and enzyme-linked immunosorbent assay (ELISA) for mycotoxins and their application for analysis of these toxins in military foods;
(e) investigation of the in vitro and in vivo neutralization of mycotoxins and STX by antibody;

(f) immunohistochemical studies on T-2 toxin;
(g) attempts to elicit monoclonal antibody against selected trichothecenes;
(h) preparation and deliver hapten, antibody, and enzyme-linked toxin to the US Army Medical Research Institute of Infectious Diseases(USAMRIID).

During the contract period, our efforts have been focused on the development of efficient methods for the production of antibodies against several important trichothecenes. Efforts for the production of antibody against metabolites of T-2 toxin and diacetoxyscirpenol (DAS) have also been made. In addition, we have developed a new method to prepare stable T-2 and DAS-protein conjugates. Using different approaches, we have successfully produced useful antibodies with different specificities against T-2 toxin, HT-2, 3'-OH-T-2, T-2 tetraol tetra-acetate, DAS, deoxyverrucarol (DOVE), acetyl-deoxynivalenol (DON), and saxitoxin. Properties of such antibodies were investigated in detail. We have also developed an efficient method for the production of 3'-hydroxyl derivatives of T-2 and T-2 toxin metabolites, as well as effective methods for the preparation of various radioactive trichothecenes. With the availability of such antibodies, studies to optimize different immunoassays, including both radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) of such toxins in different matrices have also been carried out. Studies for the application of such methods including those such as the use of RIA for monitoring T-2 and DAS produced by selected Fusaria and the use of antibodies for immunohistochemical stain of T-2 toxin and DAS in animal tissues were performed. Saxitoxin antibody also has been tested for its effectiveness in neutralization of the toxic effect in mice. The essence of these studies are presented in this report. Details of each individual study are documented in 20 manuscripts which are included as an appendix to this report.

II. WORK PERFORMED DURING THE CONTRACT PERIOD

A. Development of methods for conjugation of trichothecenes and STX to proteins and enzymes and methods for the preparation of radioactive ligands:

1. Preparation of T-2 toxin derivatives and subsequent conjugation to proteins:

Approaches that have been used for the conjugation of T-2 toxin and its metabolites to protein are summarzied in Fig. 1. At the beginning of the present contract, only one approach was tested for the conjugation of T-2 toxin to protein. T-2 toxin was converted to its hemisuccinate (HS) which was then conjugated to protein/enzyme by either a water soluble carbodiimide method or a mixed anhydride method. Several new approaches were developed during the contract period. The new approaches included conversion of T-2 toxin to the following derivatives before coupling to protein: (a) hemiglutarate (HG) of T-2 toxin; (b) O-carboxymethyl oxime (CMO) of T-2 toxin: T-2 toxin was first converted to the 3 oxide which is then converted to the CMO-T-2 toxin; and (c) hemisuccinate of 3-acetylneosolaniol (3-Ac-NEOS-HS).

2. Method for conjugation of metabolites of T-2 toxin to protein:

Considerable effort was made to develop efficient methods for the production of major T-2 toxin metabolites before coupling them to the proteins. For example, we developed a new approach for the production of large quantities of 3'-OH-T-2 toxin. Our approach included incubation of T-2 toxin with S-9 fraction isolated from livers of rats and pigs which had been treated with phenobarbitol, together with NADPH regenerating system and diethyl-p-nitrophenol (DENP, an esterase inhibitor). In the presence of DENP, T-2 toxin metabolism was completely shifted to the hydroxylation route. As much as 83% of the T-2 toxin was converted to 3'-OH-T-2 toxin. Using the same approach, 3'-OH-HT-2 and 3'-OH-acetyl-T-2 toxin were prepared with a conversion yield in the range 72-82%. A hemisuccinate of 3'-OH-T-2 was prepared and conjugated to bovine serum albumin (BSA) for immunization. Details of this study are described in publication 10.

Conjugation of HT-2 toxin and T-2 tetraol (T-2-4ol) to protein was achieved by first converted T-2 toxin to CMO-T-2 toxin which was then subjected to partial hydrolysis or completed hydrolysis to yield the CMO derivatives of HT-2 and T-2 tetraol. Acetylation of CMO-T-2 tetraol resulted in triacetyl-CMO-T-2. These CMO derivatives were then conjugated to BSA for immunization (publication 18).

3. Methods for conjugation of DAS to protein and enzymes:

The approaches that have been used for conjugation of DAS to proteins are similar to those for T-2 toxins. Thus, the toxin was first con· ·ted to its HS, HG, and CMO derivatives which were then coupled to BSA to form DAS-HS-BSA, DAS-HG-BSA and CMO-DAS-BSA conjugates.

4. Methods for conjugation of DOVE to proteins and enzymes:

For DOVE, a hemisuccinate was prepared according to a method similar to the preparation of T-2-HS and was conjugated to BSA via the water soluble carbodiimide method.

5. Methods for conjugation of deoxynivalenol (DON) to proteins and enzymes:

The following DON derivatives were prepared and subequently conjugated to BSA, polylysine, and peroxidase by either water soluble carbodiimde method or mixed anhydride technique : (a) carboxymethyl oxime (CMO) of DON; (b) triacetyl(TA)-DON; (c) TA-8-OH-DON; (d) TA-8-HS-DON; (e) TA-CMO-DON; (f) thioglycolic-DON; (g) DON-3-HS; (h) DON-3-HG; (i) 15-aldehyde DON and (j) HS of 7,8 dihydoxycalonectrin (DHC). CMO-DON was prepared by reaction of DON with carboxymethoxyl-amine.HCl under alkali conditions. Compounds (b) to (e) were prepared after converting DON to its triacetate and reducing it to the 8-OH derivatives or converting to the TA-CMO-DON. Thioglycolic-DON was preparedby direct rection thioglycolic acid with DON in the alkline condition. DON-3-HS and DON-3-HG were prepared in three steps; the hydroxyl groups at 7 and 15 positions were first protected by formation of a benzylidene(BZ)-DON which was then reacted with succinic or

glutaric anhydride; and finally, the benzylidene groups were removed from the 7 and 15 position. 15-aldehyde-DON was prepared by mild oxidation and characterized by mass spectral analyses (C.I. and E. I.). Details for the preparation of these derivatives were given in previous quarterly reports.

All the above derivatives were conjugated to the BSA (CMO also conjugated to ovalbumin) via either the water soluble carbodiimide method or mixed anhydride method, and subequently used for immunization. Antibody titers as measured by either direct ELISA or by an indirect ELISA were demonstrated in most rabbits immunized with the immunogens. However, with the exception of the last derivative, i.e. HS-DHC (compound j), the antibodies reacted most effectively with the DON-protein conjugate but not with free DON or free triacetyl-DON. Considerable efforts were made to characterize the antibody produced against HS-DHC and the details of this study were described in publication 13.

6. Methods for conjugation of STX to proteins:

For saxitoxin, the following compounds were prepared: (a) STX-HS prepared by succinylation of reduced STX (saxitoxinol or STXOL, which was prepared by reduction with $NaBH_4$, with succinic anhydride; (b) decarbamoyl-STX (DEC-STX) prepared by hydrolysis of STX in the presence of 6N HCl. Both derivatives were subsequently conjugated to BSA for immunization. Saxitoxin was also conjugated to BSA (STX-HCHO-BSA) by cross-linking with formaldehyde (publication 6).

7. Preparation of tritiated trichothecenes:

Because marker ligand with a highly specific radioactivity plays a key role in the development of a RIA for a substance to be analyzed, we have devoted considerable effort developing an efficient method for the preparation of radioactive trichothecene mycotoxins. Methods for the preparation of highly specific tritiated DAS, DON, acetyl-DON, DOVE, and T-2 toxins were developed. The principals involved the oxidation of the hydroxyl group in the toxin molecules under selected conditions and then reduction with highly specific tritiated $NaBH_4$ (78 Ci/mmole).

(a) T-2 toxin and T-2 toxin metabolites: Tritiated T-2 toxin was prepared after converting T-2 toxin to T-2-oxide which was then reduced with undiluted tritiated $NaBH_4$. The highly specific tritiated T-2 toxin (19.5 Ci/mmole) was used to prepare all other different T-2 toxin metabolites by either chemical or enzymatic method as described above.

(b) DAS: DAS was prepared using a procedure similar to that described for T-2 toxin after converting DAS to the DAS-3-oxide and subsequently reduction with $NaBH_4$.

(c) DOVE and verrucarin A (VA): For tritiated DOVE, 15-aldehyde-DOVE, prepared by oxidation of DOVE with CrO_3.2 pyridine in dry methylene chloride, was reduced with high specific tritiated $NaBH_4$ (78 Ci/mmole). A tritiated VA was prepared by reduction of 2-dehyro-VA with tritiated $NaBH_4$.

(d) DON and acetyl DON: A radioactive DON was prepared by oxidation of the CH_2OH at the C-15 position to 15 aldehyde-DON and then reduction to DON tritiated $NaBH_4$ (sp. activity 79.1 Ci/mmol) under very mild conditions (9% of tritium to DON and 2% of tritium to 8-OH-DON) . Tritiated acetyl-DON was prepared by acetylation of ^3H-DON with acetic acid anhydride in the presence of pyridine and then purified by TLC.

(e) Saxitoxinol (STXOL): STXOL was prepared by reduction of STX with tritiated $NaBH_4$ according to the method of Koehn et al. (Bioorgan. Chem. 10:412, 1981).

B. Production and characterization of specific antibodies against trichothecenes and STX:

1. Production of antibody against T-2 toxin:

(a) Improvement for the production of antibody in rabbits: A systematic study for the factors affecting the production of antibody against T-2 toxin in rabbits was carried out. Both T-2 hemisuccinate (T-2 HS) and T-2 hemiglutarate (T-2 HG) were used as immunogens in the test. The results indicate that: (i) T-2 HS-BSA was a better immunogen than T-2 HG-BSA; however, high variation of the antibody titers was observed among the rabbits which were immunized with T-2 HS-BSA; (ii) immunizations with conjugates containing moderate amounts of T-2 toxin per mole of BSA, i.e., 10-15 moles of T-2 toxin per mole of BSA resulted in best antibody titers; (iii) the optimal booster injection time was found to be about every 5-7 weeks; (iv) high antibody titers (14,000) were obtained from the rabbits 50 weeks after initial immunization, with 6 booster injections.

(b) Routine production of antibody against T-2 toxin: For routine antibody production, 7 rabbits were immunized with either T-2-HS-BSA (4 rabbits) or T-2-HG-BSA (3 rabbits) conjugates with booster injection every 5 weeks throughout the contract period. Maximum antibody titer for these rabbits after each boost were around 8,000-14,000. Most of the antibodies delivered to USAMRIID were prepared by this approach.

(c) Immunization of new T-2 toxin-protein conjugates: Three groups of rabbits (6 each) were immunized with immunogens which were prepared by conjugation of "a"-CMO- T-2 toxin ("a" isomer), "b"-CMO-T-2 toxin and 3-Ac-NEOS-HS to BSA. The antibody titers for rabbits after immunization with different T-2 toxin immunogens and the cross-reactivities of the antibodies with various trichothecenes are summarized in table 1. Antibodies against both isomers were demonstrated as early as 4 weeks after immunization. The "a"-CMO-T-2-BSA conjugate was found to be a better immunogen than the "b" isomer. In general, the specificity of antibodies obtained from rabbits after immunizing with CMO-T-2-BSA was similar to that of T-2-HS-BSA or T-2-HG-BSA. The anti-"b"-CMO-T-2 antibodies had slightly higher cross-reactivity with HT-2 toxin as compared with the antibody obtained from rabbits immunized with the conjugate of the "a" isomer. With the exception of anti-3-Ac-NEOS, the overall results indicate that most of these antibodies are specific for T-2 toxin (publications 11 and 20).

2. Production of antibody against T-2 toxin metabolites:

(a) 3'-OH-T-2 toxin: Antibody against T-2 toxin cross-reacted with HT-2 toxin to some degree, weakly with 3'-OH-T-2 toxin but poorly with T-2 tetraol. Efforts to raise antibody against these three major T-2 toxin metabolites were made. Antibodies against 3'-OH-T-2 toxin were demonstrated by a RIA 10 weeks after immunizing rabbits with 3'-OH-3-HS-T-2-BSA, using tritiated 3'-OH-T-2 toxin as the test ligand. Highest titers (6,000) were obtained 17 weeks after immunization and two booster injections. The antibodies showed good cross-reactivity with T-2 toxin, acetyl-T-2 toxin, 3'-OH-acetyl-T-2 toxin. Results of this study were described in publication 12.

(b) HT-2 and T-2 tetraol tetraacetate (T-2 4-Ac): Antibodies against HT-2 toxin and T-2 4-Ac were obtained from rabbits 5-10 weeks after immunizing the animals with CMO-HT-2-BSA and triacetyl-CMO-T-2-BSA conjugates. Immunization with CMO-T-2 4ol-BSA resulted in no antibody against T-2 4ol. The antibody produced against HT-2 toxin had great affinity for HT-2 toxin as well as good cross-reactivity with T-2 toxin. Antibody against triacetyl-CMO-T-2 was found to be very specific for T-2 4Ac and had less than 0.1% cross-reactivity with T-2 toxin, HT-2 toxin, acetyl T-2 toxin, DAS, DON, and acetyl-DON as compared to T-2 4Ac. The antibody titers for rabbits after immunization with various T-2 toxin metabolites conjugated to BSA and the cross-reactivities of these antibodies with various trichothecenes are summarized in table 2. Details of this study were described in publication 18.

3. Production of antibody against other trichothecenes:

(a) Diacetoxyscirpenol (DAS): Three approaches were tested for production of antibodies against DAS. These included immunization of rabbits with DAS-HS-BSA, DAS-HG-BSA and "b"-CMO-DAS-BSA conjugates. Among these, DAS-HS-BSA was found to be a poor immunogen. Useful antibodies were obtained from rabbits after immunization with the other two conjugates. The cross-reactivity of the anti-DAS antibodies with different trichothecenes are given in table 3. Whereas the anti-DAS-HG-BSA showed good specificifity with DAS and only had weak cross-reactivity with other metabolites, the "b"-CMO-DAS-BSA antibody did show a high degree of cross-reactivity with monoacetoxyscirpenols (MAS). The affinity constants of anti-"b"-DAS-BSA to DAS, 4-MAS and 15 MAS were found to be 4.89, 1.09, 0.87 x 10^7 liters/mole, respectively. On the other hand, the affinity constants of anti-DAS-HG with DAS, 4-MAS and 15-MAS were found to be 4.23 x 10^9, 1.15 X 10^6 and 1.44 X 10^6, respectively. Because of their cross-reactivity, unlike the anti-HG-DAS-BSA antibodies, the anti-"b"-CMO-DAS antibodies could be used for immunoassays of MAS. Details of these studies were described in publications 7 and 11.

(b) Verrucarin A (VA) and deoxyverrucarol (DOVE): The antibody against DOVE was produced by immunizing rabbits with DOVE-HS-BSA conjugate. The antibodies bound with either tritiated DOVE or diacetoxyscirpenol (DAS), but not with tritiated T-2 toxin. The affinity of antibodies with DOVE was found to be much higher than DAS. The cross-reactivity of this antibody with other related trichothecenes are

shown in Table 3. Details of this study were described in publication 8. An attempt for the production of antibody against VA was also made. VA conjugated to BSA after conversion to its hemisuccinate and was then used as the immunogen. Tritiated VA was used as a marker ligand to monitor the antibody titer. However, no antibody titer was demonstrated 12 weeks after immunization.

(c) Deoxynivalenol (DON) and acetyl-DON: Antibodies against DON-triacetate (Acetyl-DON or Ac-DON) were prepared by immunization of rabbits with the hemisucciate derivative of 7,8 dihydroxycalonectrin (DHC) conjugated to BSA. Using tritiated Ac-DON as the testing ligand, antibody titer was demonstrated as early as 4 weeks after immunization. Useful antibody for radioimmunoassay of Ac-DON was obtained from the rabbits 7 weeks after immunization, with one booster injection. Competitive RIA revealed that the antibody was most specific to Ac-DON and cross-reacted weakly (less than 0.3% as compared with Ac-DON) with T-2 tetraol tetraacetate, 15 acetyl-DON and acetyl-T-2 toxin. Practically no cross-reaction was found with DON, diacetoxyscirpenol, nivalenol and T-2 toxin. Details of this study were described in publication 13.

4. Attempts to develop a generic-type of antibody against trichothecenes:

Two attempts were made to produce a generic-type antibody against trichothecenes. In the first attempt (publication 8), deoxyverrucarol (DOVE), a trichothecene which contains only one -OH at the C-15 position of the trichothecene ring, was converted to its hemisuccinate and then conjugated to BSA for immunization. However, the antibody elicited by this immunogen was highly specific for DOVE rather than for a common trichothecene backbone (Table 3). Most recently, we have succeeded in obtaining a generic-type of antibody for group A trichothecenes after immunization of rabbits with 3-Ac-NEOS-HS-BSA. The antibody showed good cross-reactivity with T-2 toxin, acetyl-T-2 toxin (100%), 3'-OH-T-2 (50%) and DAS (14%); but little cross-reactivity with H-T-2, T-2 triol, T-2 tetraol, and group B-type trichothecenes. Thus, this antibody should be very useful as an immunochemical reagent for screening the important toxic type A trichothecenes. In addition, it can also be used for the determination of T-2 toxin and DAS metabolites after converting them to the acetylated derivatives. Details of this study were described in publication 20.

5. Production and characterization of antibody against saxitoxin:

Three immunogens, i.e. STX-HS-BSA, decarbamoyl-STX (DEC-STX-BSA), and STX-HCHO-BSA, were tested for their efficiency in production of antibody against STX. An indirect ELISA was used to monitor the antibody titer. Whereas antibody titers against the immunogens were demonstrated, only those obtained from rabbits which had been immunized with STX-HCHO-BSA were useful for ELISA. Antibodies against STX were demonstrated in rabbits by an indirect ELISA as early as 5 weeks after they were immunized with 500 ug of STX-HCHO-BSA. Competitive indirect ELISA for the STX-HCHO-BSA antibody revealed that the antiserum did not cross-react with either carbamoyl-neo-STX-sulfate or tetrodotoxin. The

antibodies cross-reacted with decarbamoyl-STX and neo-STX at about 56% and 16% those for STX. The lower detection limits for STX, decarbamyol-STX and neo-STX in this system were around 25, 45, and 156 pg per assay, respectively. Details of this study were described in publication 5.

6. Attempt to produce monoclonal antibody against T-2 toxin:

As an initial approach for the production of monoconal antibody against T-2 toxin, the conditions for producing antibody in Balb/c mice were studied. Both the amount (2.5,5 and 10 ug per mice) and type of immunogens (T-2 HS-BSA, T-2 HG-BSA, & T-2-HS-hemocyanin) used in the immunization were investigated. Hihgest antibody titers against T-2 toxin were obtained from mice which have been immunized with 10 ug of T-2 HS-BSA (12 moles/mole of BSA) per mouse (multiple sites injections were made for the initial injection), 5 days after the second booster injection (i.p. injection for the boosters) which was made 14 weeks after the initial immunization.

Monoclonal antibodies against T-2 toxin were obtained through a collaborative study with Dr. Hunter of the Uniformed Service University in Washington, D.C. Several T-2 toxin and DOVE conjugates (T-2 HS conjugated to goat IgG, hemocyanin and T-2 polylysine; DOVE-HS to hemocyanin) were sent to Dr. Hunter for immunization in mice in an immunomodulation system in which the mice were injected with T-2-HS-IgG together with goat antimouse IgD. An indirect ELISA described in one of our publications (No. 5) was used to monitor the antibody titer. However, we found that the affinity of the monoclonal antibody produced was about one log less than the polyclonal antibodies (1.75×10^{10} liter/mol for polyclonal vs 5.8×10^{7} liter/mol for monoclonal). The monoclonal antibody had higher affinity to HT-2 than T-2 toxin. Details of this study were described in publication 9.

C. Development of protocols for immunoassays:

1. Radioimmunoassay for different trichotheceneS:

(a) Sensitivity of radioimunoassay for different trichothecenes: The sensitivity of RIA for different trichothecenes with various antibodies in the buffer solution is summarized in table 4. It is apparent that in the absence of sample matrices and with high specific tritiated ligand, the RIA can detect less than 0.1-0.5 ng of toxin in each assay. In general, the specific radioactivity of the toxin and antibody quality greatly affect the sensititivty of the assay. Most of the RIAs were done by using an ammonium sulfate precipitation method to separate the free and bound toxin. An improved RIA for the assay of T-2 toxin, using albumin-coated charcoal to separate the free and bound toxin, was developed by USAMRIID through a colloborative study (publication 1).

(b) RIA of DON in corn and wheat: With the availability of antibody against DON-TA, a RIA for DON in wheat and corn was established. The DON was extracted from the sample with acetonitrile:water (84:16), defatted with hexane and then reacted with acetic anhydride to form DON-TA. The reaction mixture was subjected to a

Sep-Pak C-18 cartridge treatment to remove excess reagents and impurities. Acetylated DON was eluted from the cartridge with 50% methanol in water and then subjected to RIA where antiserum prepared against DON-TA and tritiated DON-TA were used. The overall recovery for DON added in wheat between 50-5000 ppb was 86% with a standard variation of 7% and variation coeffecient of 8%. The minumal detection for DON was around 20 ppb. The application of this method to limited naturally contaminated wheat, corn and mixed feed samples (12 samples) were tested. The RIA results for those samples were generally agreed with those obtained from TLC, analyzed by other laboratories. Since samples which contained DON without acetylation gave negative results, the present method can also serve as an confirmatory test. However, because final results are presented as DON-TA, any other mono-acetyl-DON, e.g. 3-acetyl-DON, or 15-acetyl-DON, and diacetyl-DON is also detectable. Thus, for a naturally-contaminated sample, the DON level detected by the present method will be the overall total DON, which contains DON as well as other acetylated DON (publication 16).

2. Enzyme-linked immunoassays for trichothecenes:

(a) Indirect ELISA for T-2 toxin: An indirect ELISA which can detect 0.2 to 1 ng of T-2 toxin per ml in urine, serum and milk was developed. T-2-HS was conjugated to polylysine which was then coated to a microtiter plate and incubated with rabbit anti-T-2 antibody and sample extract. The amount of anti-T-2 antibody bound to the plate was then determined by reaction with goat anti-rabbit IgG-peroxidase conjugate and by subsequent reaction with the substrate. Samples spiked with T-2 toxin were subjected to a simple cleanup procedure by passing them through a reversed-phase Sep-Pak cartridge (C-18). The recoveries of tritiated T-2 toxin added to the urine, serum and milk samples were between 71-90% after the cleanup step. In the ELISA, significant interference was observed when more than 5 ul of sample, without cleanup treatment, were used in each analysis. After cleanup, extracts equivalent to 50 ul of urine, serum or milk per well did not significantly interfere with the assay. The recoveries of T-2 toxin added to serum (1 to 10 ng/ml), urine (0.2 to 10 ng/ml) and milk (0.2 to 10 ng/ml) after cleanup treatment as determined by the indirect ELISA were found to be 51 to 82%, 73 to 82%, and 80 to 83%, respectively. Details of this study were described in publication 5.

We also found that the indirect ELISA can be carried out by coating the ELISA plate with the original immunogen, such as T-2-HS-BSA. However, the primary antibody should be pre-incubated with a modified BSA solution which was prepared by reaction of BSA with the coupling reagent in the absence of T-2-HS.

(b) Simultaneous determination of T-2 toxin and HT-2 toxin by an indirect ELISA: An indirect ELISA for the detection of HT-2 toxin in the presence or absence of T-2 toxin was also developed. In this assay, the relative cross-reactivity of anti-T-2 antibody (T-2-HS) with T-2 toxin and HT-2 toxin was found to be 1 and 0.1, whereas anti-HT-2 antibody (CMO-HT-2) with T-2 toxin and HT-2 toxin was found to be 0.33 and 1, respectively. Using such relationships, a formula was established which could be used to calculate the individual toxin

concentration in a mixed sample after experimentally analyzing for T-2 and HT-2 toxins in the two separate indirect ELISAs. This method was tested by analyzing urine samples which had been spiked with HT-2 toxin alone and samples spiked with both T-2 toxin and HT-2 toxin. A clean-up protocol for the treatment of urine samples before ELISA was also established. The overall analytical recovery of HT-2 toxin when added at a concentration of 0.1 to 10 ppb to the urine samples was around 89%. When both T-2 and HT-2 toxins were added to the urine samples at equal concentrations of 0.5 to 5.0 ppb, the recoveries for T-2 and HT-2 toxins were found to be 112% and 109%, respectively. Details of this study were described in publication 19.

(c) Development of immunoassay for DAS: We have tested both direct and indirect ELISA for DAS. In the direct ELISA, the diluted antiserum was coated to the plate by the glutaraldehyde method. Both DAS-hemiglutarate (HG)-horseradish-peroxidase (HRP) and DAS-hemisuccinate (HS)-HRP conjugates were tested for their ability to bind with the antiserum. DAS-HG-HRP was found to be more effective than that DAS-HS-HRP preparation. The ELISA permits detection as low as 25 pg of DAS in each assay (1 ng/ml and 25 ul used in each assay). However, because of interfering substances in the sample matrix, the minimal detection levels for DAS in wheat and corn by RIA and ELISA were found to be 25 and 50 ppb, respectively. In the indirect ELISA, "a"-CMO-DAS-BSA was used as the antigen coated onto the microELISA plate using the protocols similar to those for T-2 toxin. The non-specific binding of antibody to the antigen was blocked by incubation with a modified BSA solution. The sensitivity of this assay was found to be around 5 pg per assay (0.1 ng/ml, 50 ul sample each assay) in buffer solution.

3. An indirect ELISA for STX and STX antibody:

An indirect ELISA, using the same protocols as those for T-2 toxin, was used for the detection of STX and STX antibody. In this assay , STX-BSA conjugate (STX-HCHO-BSA) or polylysine-STX was precoated onto the microELISA plate. The lower limit for detection of STX by the indirect ELISA was around 25 pg per assay. When STX spiked to the clams or mussels was tested, the detection limit for STX was around 50-100 ppb with recoveries in the range of 86.8-106.5% (publication 5).

D. Development of an immunohistochemical method for detection of trichothecenes:

1. The immunoperoxidase localization of T-2 toxin:

The fate of T-2 toxin in mice after receiving a single oral dose of 11 mg/kg was monitored by the peroxidase-antiperoxidase (PAP) method. This method was more sensitive and needed less antiserum to produce an optimal stain density than the direct immunohistochemical stain in which an enzyme is conjugated to a specific antibody. The slides were first treated with anti-mycotoxin antibody, then incubated with goat antirabbit-IgG, and finally incubated with the (PAP) complex before being stained with the substrate. T-2 toxin was demonstrable in the esophagus as early as 15 min until about 24 h post-dosing. In the

stomach, T-2 toxin was detected within the cytoplasm of intact and injured epithelial cells. In the duodenum, T-2 toxin was primarily localized within the surface epithelium and phagocytic elements (macrophage and neutrophils) of the duodenal lamina propria, especially toward the tips of the villi. Following sloughing of duodenal villous tips, the recovered villous tip epithelial cells frequently showed both cytoplasmic and nuclear T-2 toxin. The jejunum showed weak T-2 toxin within the cytoplasm of villous tip epithelial cells only. The ileum never demonstrated T-2 toxin. Contrary to observations for ochratoxin A, the kidney medulla contained the majority of T-2 toxin stain. T-2 toxin was never demonstrable in any of the hepatic tissue examined. This observation was not suprising because hepatocytes metabolize T-2 toxin rapidly to HT-2 and other metabolites, which are not very reactive with the antibodies used in this study. Details of this study were described in publication 2.

2. DAS studies:

Different organs obtained from mice at various times after feeding with DAS at a dose of 10 mg/kg were examined for pathological lesions as well as for immunohistochemical stains according to the PAP method described for T-2 toxin. Preliminary data showed that no significant lesions were found in the organs of the mice fed at this dosage.

E. Neutralization of the toxic effect of STX and T-2 toxin antibodies:

1. Neutralization of T-2 toxin with antibody against T-2 toxin:

Passive immunization of CF-1 mice with rabbit anti-T-2 antiserum (anti-T-2-HS-BSA) was carried out. In a preliminary experiment, the levels of circulating antibody and its persistence after passive immunization with 0.5 ml of anti-T-2 antiserum (titer 5,000) without challenge with T-2 toxin was determined. We found that antibody titer was detected at 3 h post-injection with the antiserum. Levels remained constant from 24-96 hrs at a titer (RIA) approximately 10% of that injected. Detectable levels of antibody persisted through 144 hours post-injection. However, when challenged with T-2 toxin, no significanct difference between the passive immunized mice and the control mice on the course of T-2 toxicosis, as determined by the LD^{50}, circulating leukocytes, and serum alkaline phosphatase, was observed. Using tritiated T-2 toxin, we also did not find any significant difference on the distribution and clearance of T-2 toxin in the passive immunization group. Because only a small amout of antiserum was used in the present study, the results indicate that a large amount of high titer antiserum may be necessary for protection.

2. Neutralization of toxicity of STX with antibody in mice:

To test the effectiveness of neutralization of toxicity of STX by antibody, strain CF-1 mice , 3 per group, were each injected with one ml of different dilutions of antiserum obtained from a rabbit 8 weeks after immunization with STX-BSA. One day after injection of antisera, the mice were each challenged with 0.35 ug of STX by an i.p. injection,

and the time of death for each mouse was recorded. The results as showed that there was a slight protection for the mice pretreated with antiserum at a one to 10 dilution. Complete protection was observed for the mice preinjected with a one ml of one to 5 dilution of antiserum.

F. Production of T-2 and DAS by different Fusaria by RIA:

1. Effect of temperature on the production of T-2 toxin and DAS:

Production of T-2 toxin and DAS by two Fusarium sporotrichioides species (one previously identified as F. tricinctum) at four different temperatures (7, 15 C, 24 and 28C) in a glucose-soya meal-corn steep liquor medium absorbed in vermiculite was studied. After incubation for an appropriate time, the toxins were extracted from the culture with methanol, subjected to a Sep-Pak C-18 cartridge treatment, and then were analyzed by RIA. Fusarium tricinctum was found to be primarily a T-2 toxin producer, as little or no DAS was found in the culture medium. On the contrary, F. sporotrichioides produce both toxins; the amount of DAS was three times higher than that of T-2 toxin. A higher temperature (28C) was more favorable for DAS production whereas in contrast, a higher yield of T-2 toxin was obtained at lower temperature (15C). Maximum toxin yield was obtained 20-30 days after incubation at both temperatures. At 28 C, large amounts of DAS (600-700 mg/liter) were produced by F. sporotrichioides in each culture whereas less than 200 mg of T-2 was obtained after 25 days of incubation. At 15C, the DAS and T-2 levels produced by this fungi were found to be around 210 mg and 234 mg per liter, respectively. For F. tricinctum, between 740-900 mg and 630-740 mg of T-2 toxin per liter were produced after 20-30 days of incubation at 15C and 24C, respectively. Less than 11 mg/liter of DAS was produced by F. tricinctum under these conditions. Our results are consistent with earlier data which was obtained by GLC analysis. In addition, our results also show the potential health hazard of some Fusaria species that produce more than one type of toxic trichothecene under field conditions.

2. Studies on the production of T-2 toxin and DAS by different strains of Fusaria:

The production of T-2 toxin and DAS by 18 Fusaria in the demium described above at 15C and 24C over a period of 35 days was studied. The cultures were supplied by Dr. Nelson of Penn. State University. Among 13 strains of Fusarium sporotrichioides tested, 4 were found to be high T-2 toxin producers (greater than 700 mg of T-2/liter at 15C and 200 mg/L DAS at 24C), 2 were high DAS producers (700-900 mg/L DAS at 24C; 200 mg/L T-2 at 15C); 3 strains produced both toxins in good yield (300-600 mg of both toxin at 24 or 15C), 3 were identified as low toxin producers (less than 300 mg/liter) and one did not produce T-2 toxin and DAS. T-2 toxin or DAS were not detected in the medium inoculated with 3 strains of F. graminearum and two F. chlamydosporum.

III. DISCUSSION AND ASSESSMENT OF WORK DONE

During the contract years, rapid progress in the area of immunoassays for trichothecene mycotoxins was made in our laboratory.

Considerable progress in improving the production of antibody against T-2 toxin in rabbits were made. High titers of antibody were obtained from rabbits after prolonged repeated immunization with T-2-HS-BSA conjugates which contained 10-12 moles of hapten per mole of BSA. Several new T-2 conjugates were prepared and proved to be good immunogens. The effect of chain length between T-2 or DAS and BSA on the immunogenic properties of the conjugates can be seen from the rabbits' response to different conjugates. However, different effects were observed with these two toxins. Whereas T-2 HS-BSA appears to be better than T-2 HG-BSA for antibody production against T-2 toxin, DAS-HG-BSA was found to be better than DAS-HS-BSA. Although this difference might be due to a difference in the orientation of the mycotoxins around the protein molecule, the stability of the conjugates may also play a role. If the poor immunogenic properties of these conjugates is due to an in vivo hydrolysis of the hapten from the protein molecule, then, the rate of hydrolysis of the hemisuccinates and hemiglutarates may directly affect the efficiency of these conjugates for antibody production. The hydrolysis problem was overcome by immunization of rabbits with conjugates of the CMO derivatives. Although such derivatives are very stable and showed a rapid response in eliciting antibody, they also did not produce high antibody titers in a prolonged immunization schedule. The only immunogen that gave a rapid immune response for T-2 toxin was 3-Ac-NEOS-HS-BSA, which was conjugated to the protein at the C-8 position, a side chain which plays an important role for protein synthesis inhibition. Such results support our earlier hypothesis that immunosuppression also may play an important role for eliciting antibody against trichothecenes.

With our supply of immunogens and other test reagents, a monoclonal antibody against T-2 toxin was obtained in Dr. Hunter's laboratory. However, the antibody was more specific to HT-2 toxin than T-2 toxin. The affinity of the polyclonal antibody was also higher than the monoclonal antibody. Thus, efforts to produce high affinity monoclonal antibody for T-2 and related toxins should be continued.

A number of antibodies against T-2 metabolites as well as other trichothecene mycotoxins were produced. The properties of these antibodies were studied in detail. Results obtained from the cross-reactivities of these antibodies with different trichothecenes (Tables 1-3) indicate that each antibody has its own specificity. As more data were accumulated, we found that the side chain of trichothecene groups played an important role in eliciting antibody against this group of mycotoxins. Therefore, it was possible to produce antibodies against several types of mycotoxins within the trichothecene group through modification of the side chains. In this regard, we have made antibody against acetyl-DON and T-2 tetraol tetra-acetate. These antibodies will be useful for detection of DON as well as T-2 tetraol. Whereas efforts that have been made to produce a generic antibody attempting to detect all types of trichothecenes were unsuccessful, an antibody that can recognize type A trichothecene has been obtained after immunization of rabbits with 3-Ac-NEOS-HS-BSA during the last year of the contract. Such an antibody should also be useful for monitoring the metabolites of type A trichothecene mycotoxins after these metabolites are acetylated.

In the present contract, much effort was concentrated in the type A tricothecences area. However, antibodies against DOVE also show some cross-reactivity with verrucarrol. Thus, it is possible to use the DOVE antibody for the analysis of macrocyclic-type trichothecenes after removal of the macrocylic structure through hydrolysis. We have encountered difficulties in the production of antibodies against type B trichothecenes such as DON. The main problem was due the presence of many hydroxyl groups in the molecules. The presence of such groups in the molecule not only creat problems in conjugation to proteins, but also appear to render the molecule less immunogenic. Future work should be directed toward these two types of trichothecenes.

With the availability of antibodies, studies to optimize the conditions for RIA and to develop efficient ELISAs for different toxins in different sample matrices were conducted. The sensitivity of RIA was improved consideraably by using higher titer antibody preparation and also by the use of high specific radioactive T-2 toxin. An indirect ELISA for T-2 toxin was developed (publication 5) and has also been tested by USAMRIID. However, there were some problems in its reproduciblity, which might be due the instability of the T-2-HS-polylysine conjugate. Subsequent studies led to the production of CMO-derivatives of T-2 and DAS. More reproducible results were obtained when CMO-T-2-polylysine or CMO-DAS-polylysine was used. Protocols for determination of DON, STX, and simultaneous determination of T-2 and HT-2 toxin in biolgical samples by either RIA or indirect ELISA were developed. Although a simple cleanup procedure is still needed in such assays, these methods are more sensitive and simple than other chemical methods amd will be useful for analysis of such toxins. Because there was still a high degree variation in the assay, future studies for ELISA of trichothecene mycotoxins and STX should be directed to better quality control of reagents, uniformity of ELISA protocol, development of a direct ELISA system, such as the dipstick-type assay, as well as additional collaborative studies.

Radioimmunoassays developed in our laborotory for the detection of T-2 and DAS have been applied to monitor the production of these two toxins by different Fusaria species. This further showed the advantages of RIA over other analytical methods. Our results indicate that the ability of producing toxins varied considerably with the species tested, as well as temperatures and substrates used. This information will be extremely useful for our understanding of the etiology of toxin production. Studies on the modification of T-2 metabolism by esterase inhibitors not only led to a new approach for the production of some major T-2 metabolites but also unveiled the possibile impact of environmental agents on T-2 toxicity.

With the availability of different antibodies, such antibodies can also be used as immuohistochemical reagents to monitor the presence of small molecular weight toxins in tissues and organs as well as be used as a prophylactic agent to neutralize the toxic effects. Whereas we have succeeded in developing a method for monitoring T-2 toxin in different organs and tissues, we were not able to demonstrate the presence of DAS in different organs by this technique. This might be due to the low DAS antibody titer. It may also due to the rapid

metabolism of DAS to other metabolites. For example, we have never demonstrated any T-2 toxin in liver tissue. Further studies should be directed to using antibodies of different specificity, especially those for the toxin metabolites, as the histochemical reagents. Although we have demonstrated that STX antibody can neutralize the toxic effects of STX in mice to some degree, we failed to demonstrate the protective effect for T-2 toxin after administration of anti-T-2 antibody to mice. Such results led us to believe that a large amount of antibody is needed to neutralize the toxic effect.

In the development of immunoassays for dinoflagellate toxins, only STX was investigated in the present contract. We have obtained antibodies which are capable of neutralizing the toxic effect as well as being useful in the ELISA. Future study should be directed to a search for other methods of improving antibody titers as well as producing antibodies for other dinoflagellate toxins.

IV. DELIVERABLES

The immunochemical reagents prepared and delivered to the USAMRRID since the beginning of this contract are summarized in table 5.

V. LIST OF PUBLICATIONS

1. Fontelo, P.A., Beheler, J., Bunner, D. L. and Chu, F. S. 1983. Detection of T-2 toxin by an improved radioimmunoassay. Appl. and Environ. Microbiol. 45:640-643.

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13. Zhang, G. S., Li, S. W. and Chu, F. S. 1986. Production and characterization of antibody against deoxynivalenol triacetate. J. Fd. Prot. 49: 336-339.

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17. Wei, R. D. and Chu, F. S. 1986. Instability of some trichothecene mycotoxins in methanol. J. Assoc. Off. Anal. Chem. 69:902-903.

18. Fan, T. S. L., Zhang, G. S. and Chu, F. S. 1987. Production and characterization of antibodies against HT-2 toxin and T-2 tetraol tetra-acetate. Appl. Environ. Microbiol. 53:17-21.

19. Fan, T. S. L., Xu, Y. C and Chu, F. S. 1987. Simultaneous analysis of T-2 toxin and HT-2 toxin in urine by an indirect ELISA. J. Assoc. Off. Anal. Chem. (March issue).

20. Wei, R. D. and Chu, F. S. 1987. Production and characterization of a generic antibody against group A trichothecenes. Analytical Biochem. (Jan. issue)

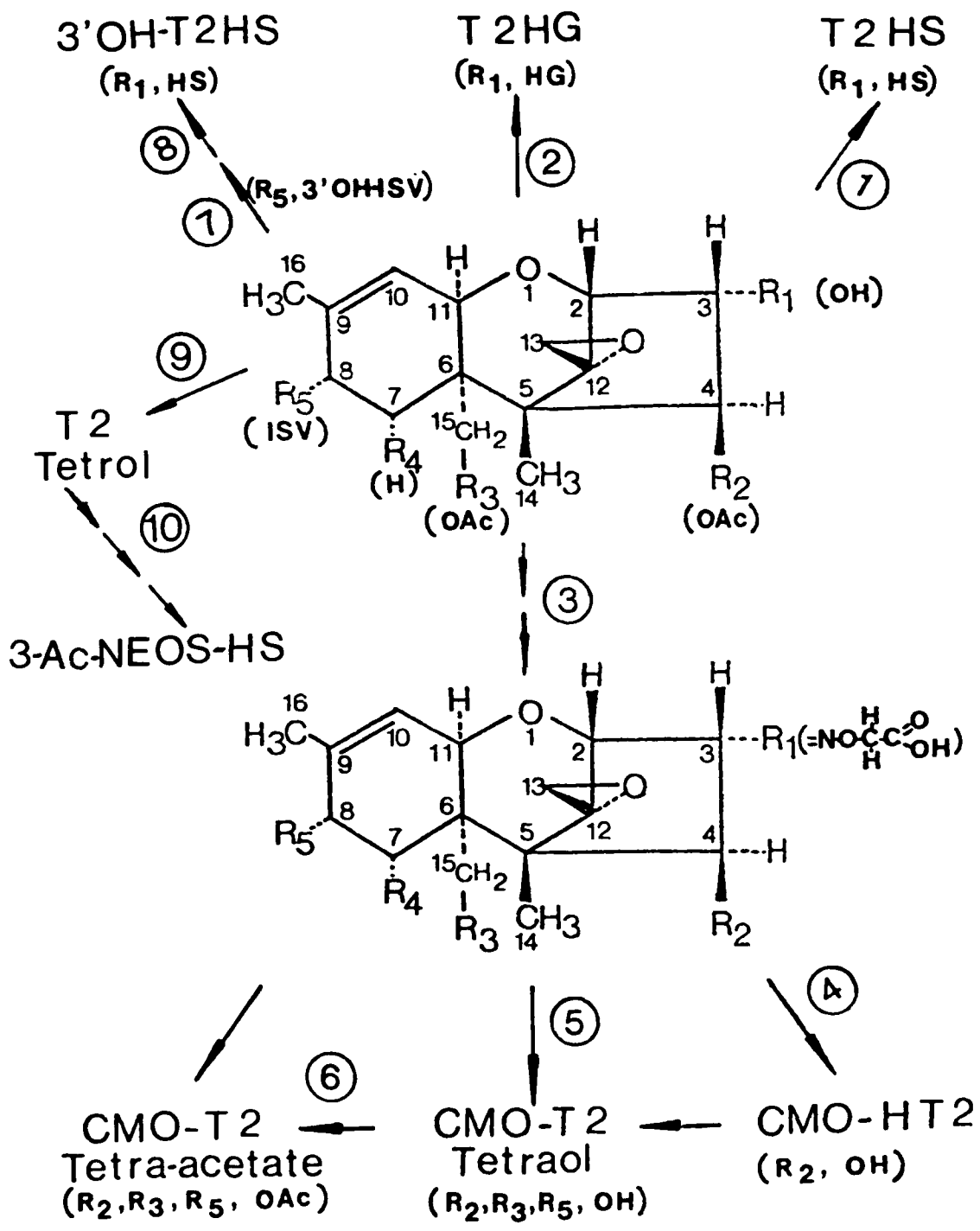

Fig. 1. Approaches used in the conjugation of T-2 toxin to protein.

TABLE 1 RELATIVE CROSS-REACTIVITY OF DIFFERENT ANTIBODIES AGAINST T-2 TOXIN WITH DIFFERENT T-2 TOXIN ANALOGS

TOXIN ANALOGS	CONJUGATES USED [a]				
	HS	HG	a-CMO	b-CMO	3-Ac-NEOS
Ac-T-2	100	4	25	33	100
T-2	100	100	100	100	100
ISO-T-2	50	n	n	n	1.4
HT-2	17	20	20	50	-
T-2 triol	2.2	<0.04	0.5	0.2	-
T-2 tetraol	0.1	-	0.04	-	-
3'-OH-Ac-T-2	2.0	-	-	-	3.4
3'-OH-T-2	0.5	-	0.23	-	50
NEOS	0.2	0.04	-	-	-
DAS	<0.04	<0.04	-	-	14
DON	<0.04	<0.04	<0.02	-	-
3-Ac-NEOS-HS	-	-	-	-	8
T-2-4ol-TA	-	-	-	-	2
3-Ac-NEOS	-	-	-	-	0.3
Ac-DAS	-	-	-	-	0.2
DON-triacetate	-	-	-	-	0.1
AB titers [b]	14	6	6	2	16
Sensitivity [c]	0.2	0.67	0.2	0.17	0.34

(a) All the derivatives were conjugated to bovine serum albumin for immunization. All data are expressed as % of reactivity relative to the respective trichothecene mycotoxins.

(b) Highest titer, in thousands.

(c) Concentration in ng/assay for compounds to displace 50% of the radioactive ligand. Generally, the compounds which had 100% binding were the marker ligands.

TABLE 2 RELATIVE CROSS-REACTIVITY OF ANTIBODIES AGAINST T-2 TOXIN METABOLITES WITH DIFFERENT T-2 TOXIN ANALOGS

TOXIN ANALOGS	CONJUGATES USED [a]			
	T-2 HS	3'-OH-T-2-HS	CMO-HT-2	T-2-4ol-4Ac
Ac-T-2	100	34	3.3	<0.01
T-2	100	25	25	<0.01
ISO-T-2	50	3.3	10	-
HT-2	17	2.2	100	<0.01
T-2 triol	2.2	<0.04	0.1	-
T-2 tetraol	0.1	<0.04	<0.06	-
3'-OH-Ac-T-2	2	20	0.08	-
3'-OH-T-2	0.5	100	0.15	-
3'-OH-T-2-HS	nd	6.7	<0.06	-
3'-OH-HT-2	nd	0.6	0.25	-
NEOS	0.2	nd	nd	-
DON	<0.04	<0.04	<0.06	<0.01
T-2-4ol-4Ac	nd	nd	nd	100
AB titers[b]	14	6	1.6	3.7
Sensitivity[c]	0.2	0.4	0.62	0.10

(a) All the derivatives were conjugated to bovine serum albumin for immunization. All data are expressed as % of reactivity relative to the respective trichothecene mycotoxins.

(b) Highest titer, in thousands.

(c) Concentration in ng/assay for compounds to displace 50% of the radioactive ligand. Generally, the compounds which had 100% binding were the marker ligands.

TABLE 3. RELATIVE CROSS-REACTIVITY OF ANTIBODIES AGAINST DIFFERENT TRICHOTHECENE MYCOTOXINS

TRICHOTHECENES	CONJUGATES USED (a)				
	T2-HS	CMO-DAS	DAS-HG	DOVE-HS	Ac-DON
Ac-T-2	100	0.7	-	-	0.1
T-2	100	1.0	<0.08	<0.01	<0.1
ISO-T-2	50	-	-	-	-
HT-2	17	-	-	-	-
T-2 triol	2.2	-	-	-	-
T-2 tetraol	0.1	-	-	-	-
T-2-4-Ac	-	-	-	-	0.3
3'-OH-Ac-T-2	2	-	-	-	-
3'-OH-T-2	0.5	-	-	-	-
DAS	<0.04	100	100	<0.01 (100)(d)	<0.1
4-MAS	-	25	1.1	-	-
15-MAS	-	20	0.5	-	-
DON	<0.04	<0.05	<0.08	<0.01	<0.1
DON-Tri-Ac	-	-	-	-	100
15-Ac-DON	-	-	-	-	0.2
DOVE	-	-	<0.08	100 (120)	-
NEOS	0.22	0.04	<0.08	-	-
NIV	-	-	0.04	-	<0.1
Verrocarol	-	-	-	0.03 (120)	-
Verrocarin A	-	-	-	<0.01	-
AB titers (b)	14	0.8	1.2	2.5	2.5
Sensitivity (c)	0.2	4.2	1.5	0.32 (60)	1.0

(a) All the derivatives were conjugated to bovine serum albumin for immunization. All data are expressed as % of reactivity relative to the respective trichothecene mycotoxins.

(b) Highest titer, in thousands.

(c) Concentration in ng/assay for compounds to displace 50% of the radioactive ligand. Generally, the compounds which had 100% binding were the marker ligands.

(d) Values in parenthesis indicate that tritiated DAS was used as the marker ligand.

TABLE 4. SENSITIVITY OF RADIOIMMUNOASSAY FOR DIFFERENT TRICHOTHECENES

ANTIBODY USED	TOXIN ANALYZED [a]	CONC. RANGE [b]
T-2-HS-BSA	T-2	0.05 - 1.0
T-2-HG-BSA	T-2	0.10 - 5.0
CMO-T-2-BSA	T-2	0.05 - 2.0
3-Ac-NEOS-HS-BSA	T-2	0.10 - 1.0
CMO-HT-2-BSA	H-T-2	0.30 - 5.0
Triacetyl-CMO-T-2-BSA	T-2-4Ac	0.05 - 1.0
3'-OH-T-2-HS-BSA	3'-OH-T-2	0.05 - 3.0
DAS-HS-BSA	DAS	0.20 - 5.0
CMO-DAS-BSA	DAS	0.20 - 20.0
DOVE-HS-BSA	DOVE	0.05 - 3.0
DOVE-HS-BSA	DAS,VA,DOVE [c]	5.0 -200
Triacetyl-DON-HS-BSA	Triacetyl-DON	0.10 - 5.0

(a) Tritiated toxin (c.a. 10,000 dpm per assay) with a specific radioactivity in the range of 8.8 -19.5 Ci per mmole was used.

(b) Log. of linear range of standard toxin in phosphate buffer shown in the center column, ng/assay.

(c) Tritiated DAS was used as the marker ligand.

TABLE 5. IMMUNOCHEMICALS DELIVERED DURING THE WHOLE CONTRACT PERIOD

REAGENT NAMES	AMOUNT	
A. Tritiated trichothecenes		
DAS	1.0 mCi	
DOVE	1.0 mCi	
T-2 TOXIN	51.0 mCi	
3'-OH-T2 TOXIN	1.0 uCi	
VERRUCARIN A	2.0 mCi	
B. Trichothecenes		
HT-2	10.0 mg	
CMO-HT-2	5.0 mg	
3'-OH-T-2	10.0 mg	
3'-OH-H-T-2	1.0 mg	
C. Toxin-protein conjugates		
DAS-HG-BSA	48.5 mg	
DOVE-HS-BSA	24.0 mg	
DOVE-HS-POLYLYSINE	2.5 mg	
DOVE-HS-IgG	13.0 mg	(*)(a)
STX-BSA	22.5 mg	
T-2-HS-BSA	239.3 mg	
T-2-HS-EDA-BSA	14.0 mg	
T-2 HG-BSA	5.0 mg	
T-2-HS-POLYLYSINE	51.0 mg	
T-2-HS-HEMOCYANINE	20.0 mg	(*)
T-2-HS-IgG	10.0 mg	(*)
CMO-T-2-BSA	5.0 mg	
CMO-T-2-POLYLYSINE	1.0 mg	
CMO-HT-2-POLYLYSINE	3.7 mg	
D. Toxin-enzyme conjugates		
DOVE-PEROXIDASE	2.0 mg	
T-2-HS-PEROXIDASE	13.5 mg	
T-2-HS-ALKINE-PHOSPHATASE	2.4 mg	
E. Antibodies		
DAS ANTIBODY	41.0 ml	
DOVE ANTIBODY	13.0 ml	
H-T-2 ANTIBODY	4.0 ml	
STX ANTIBODY	10.0 ml	
T-2 ANTIBODY (T-2 HS)	152.0 ml	
T-2 ANTIBODY (CMO-T-2)	17.0 ml	

(a) Items marked with "*" were delivered to Dr. Hunter for the production of monoclonal antibody against T-2 toxin. Ten mg each of mouse IgG T-2-tetraol, goat IgG-T-2-tetraol, rabbit IgG-T-2-tetraol, AECM-Ficoll-T-2 tetraol and Brucella abortus T-2-tetraol conjugates were also delivered to Dr. Fred Finkelman of the Uniformed Services University for a collaborative study attempting to produce monoclonal antibody against T-2 tetraol.

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